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## Effects of Temperature and Solvent Condition on Phase Separation Induced Molecular Fractionation of Gum Arabic/Hyaluronan Aqueous Mixtures

Bing Hu<sup>a, b</sup>, Lingyu Han<sup>a, b</sup>, Zhiming Gao<sup>a, b</sup>, Ke Zhang<sup>a, b</sup>, Saphwan Al-Assaf<sup>c</sup>, Katsuyoshi Nishinari<sup>a, b</sup>, Glyn O. Phillips<sup>a, b</sup>, Jixin Yang<sup>d</sup> and Yapeng Fang<sup>a, b, \*</sup>

<sup>a</sup> Hubei International Scientific and Technological Cooperation Base of Food Hydrocolloids, Wuhan 430068, China;

<sup>b</sup> Glyn O. Phillips Hydrocolloid Research Centre at HUT, School of Food and Biological Engineering, Hubei University of Technology, Wuhan 430068, China;

<sup>c</sup> Glyn O. Phillips Hydrocolloid Research Centre, Wrexham Glyndwr University, Plas Coch, Mold Road, Wrexham LL11 2AW, United Kingdom;

<sup>d</sup> School of Applied Science, Computing and Engineering, Wrexham Glyndwr University, Plas Coch, Mold Road, Wrexham LL11 2AW, United Kingdom.

\*Correspondence Author: Yapeng Fang, Email: [fangypphrc@163com](mailto:fangypphrc@163com); Tel: 86-(0)-27-59750470.

**Keywords:**

phase separation; gum arabic; solvent condition

## Abstract

Effects of temperature and solvent condition on phase separation-induced molecular fractionation of gum arabic/hyaluronan (GA/HA) mixed solutions were investigated. Two gum arabic samples (EM10 and STD) with different molecular weights and polydispersity indices were used. Phase diagrams, including cloud and binodal curves, were established by visual observation and GPC-RI methods. The molecular parameters of control and fractionated GA, from upper and bottom phases, were measured by GPC-MALLS. Fractionation of GA increased the content of arabinogalactan-protein complex (AGP) from ca. 11% to 18% in STD/HA system and 28% to 55% in EM10/HA system. The phase separation-induced molecular fractionation was further studied as a function of temperature and solvent condition (varying ionic strength and ethanol content). Increasing salt concentration (from 0.5 to 5 mol/L) greatly reduced the extent of phase separation-induced fractionation. This effect may be ascribed to changes in the degree of ionization and shielding of the acid groups. Increasing temperature (from 4°C to 80°C) also exerted a significant influence on phase separation-induced fractionation. The best temperature for GA/HA mixture system was 40°C while higher temperature negatively affected the fractionation due to denaturation and possibly degradation in mixed solutions. Increasing the ethanol content up to 30% showed almost no effect on the phase separation induced fractionation.

## Introduction

Phase separation is important for industrial applications, such as food structural design [1, 2], multiple emulsion preparation [3, 4], microencapsulation [5], and protein purification and separation [6]. Mixing two biopolymers in a solvent may result in phase separation, either associative or segregative [7]. The associative phase separation

can be a consequence of a complexation process, which is initiated at the molecular scale through electrostatic interactions between biopolymers [8]. A segregative phase separation between biopolymers results from either effective electrostatic repulsion or asymmetric biopolymer-solvent interactions [9]. Factors that could affect phase separation include: (1) biopolymer concentrations; (2) biopolymer characteristics such as molecular weight, charge, shape and conformation; (3) external conditions such as temperature, pH, ionic strength, solvent composition and mechanical field [10-12]. For example, linear biopolymers have relatively larger space occupancy, thus a lower threshold concentration for phase separation than globular proteins [13]. Biopolymers are polydisperse in nature, and their phase separations are much more complex than those of model monodisperse systems [14-16]. Consequently, phase separation of polydisperse systems is often accompanied with molecular fractionations, in which fractions of higher molecular weight prefer to stay in their own phases and those of lower molecular weights tend to coexist with the other biopolymers [12, 17-19].

Hyaluronan (HA) is a linear polysaccharide belonging to the family of non-sulfated glycosaminoglycans. It consists of 1,4-linked *N*-acetyl- $\beta$ -D-glucosamine ( $\beta$ -D-GlcNAc) and 1,3-linked  $\beta$ -D-glucuronic acid ( $\beta$ -D-GlcA) disaccharide units with molecular weight up to 6 million Daltons [20]. HA was originally recognized as an inert filling material and has been widely used in numerous pharmaceutical applications [21]. Recently, HA has been discovered to be a unique polysaccharide that exhibits an astonishing array of functions in several different biological processes [22-24].

Gum arabic (GA) is a natural exudate from *Acacia* trees of the Sahelian region of Africa, and contains branched polysaccharides and proteinaceous materials. Three major fractions of GA are arabinogalactan-protein complex (AGP), arabinogalactan (AG) and glycoprotein (GP) [25]. The AGP fraction has a molecular mass of several millions Daltons and contains about 10% proteins, which is regarded as an active component for emulsification [26, 27]. Different modification methods such as radiation induced cross-linking [28] and maturation [26] have been used to enhance the

emulsifying property of GA. The basic principle of both methods is to increase the proportion of the AGP fraction.

The methods described in this paper offer a green and facile method to fractionate polydisperse biopolymers and to concentrate functional components in order to increase their functionality such as emulsifying property [19]. A lot of experimental data and theoretical descriptions of the effects of several parameters (*e.g.*, biopolymer concentration and molar mass) on phase separation can be found in the literature [29-35]. However, there is lack of investigation on the influence of temperature and solvent condition on the phase separation. The present work was designed to study the phase separation induced fractionation of the polydisperse GA/HA aqueous mixed system, and to evaluate the effects of temperature and solvent condition on the extent of fractionation. The knowledge gained in this study identifies a green and effective method for fractionating functional components in polydisperse biopolymers and an effective condition for increasing the AGP content of GA can be chosen.

## Experimental

### *Materials*

Two gum arabic samples namely EM10 (powder form LOT 101008) and standard (STD) (kibbled form LOT 42162) were obtained from San Ei Gen F.F.I, Inc. (Japan) and STARLIGHT (France), respectively. EM10 is an enhanced gum arabic obtained by maturation treatment with a weight average molecular weight ( $M_w$ ) of  $4.07 \times 10^6$  g/mol and a polydispersity index ( $M_w/M_n$ ) of 8.0. The  $M_w$  and polydispersity index for STD gum were  $0.55 \times 10^6$  g/mol and 2.5, respectively. The molecular weight values were determined using GPC-MALLS system as described previously [26, 36]. Hyaluronan (white powder LOT 41400) was obtained from Matrix Biology Institute (New Jersey, USA). HA has  $M_w=1.68 \times 10^6$  g/mol and  $M_w/M_n=2.5$  determined using gel electrophoresis method as reported previously [37]. All the other materials were

purchased from Fisher Scientific and Sigma-Aldrich (UK) and of analytical grades.

#### *Preparation of stock solutions*

The stock solutions of EM10 (40 wt%), STD (25 wt%) and HA (1 wt%) were prepared by dispersing respective dry powders in distilled water containing 0.005 wt%  $\text{NaN}_3$  as a preservative, and were left on a roller mixer overnight to ensure complete hydration. The stock solutions were stored in a refrigerator for further use. The concentrations of all solutions are expressed as weight percentage unless otherwise stated.

#### *Gel permeation chromatography-multiangle laser light scattering (GPC-MALLS)*

Phase separation induced fractionation was evaluated by measuring the molecular distribution in the upper and bottom phases using GPC-MALLS for GA/HA mixtures which exhibited phase separation to different extents. This could be varied by controlling GA and HA concentrations. After appropriate dilution, the samples taken from the upper and bottom phases were loaded into the analytical GPC-MALLS at 25 °C. [26, 36, 38]. A Superose6 10/300GL column (GE Healthcare, USA) was used to determine the molecular parameters of GA. A series of Shodex OHpak, Shodex SB-806M HR and Shodex SB-803 HR columns (exclusion limit,  $20 \times 10^6$  g/mol and  $0.1 \times 10^6$  g/mol, respectively) were used for the separation of two samples to determine the phase diagram. The detectors used in this study included an Agilent 1100 series UV detector (Agilent Technologies, UK) operated at 214 nm, a DAWN EOS multiangle light scattering detector (Wyatt Technology Corporation, USA) operated at 690 nm, and an Optilab refractometer (Wyatt Technology Corporation, UK). A NaCl solution (0.2 mol/L) with 0.005%  $\text{NaN}_3$  filtered through 0.2  $\mu\text{m}$  Millipore filter was adopted as an eluent and delivered at a constant rate of 0.4 mL/min by a KNAUER HPLC pump K-501 (Kinesis, UK). The sample solutions were injected into the GPC-MALLS system after proper dilution and filtration through a 0.45  $\mu\text{m}$  Nylon filter. Data were collected

and analyzed by Astra 4.90.08 software (Wyatt Technology Corporation, USA).

#### *Determination of phase diagram*

The binodal boundary of the phase diagram of EM10/HA was determined by refractive index response from GPC-RI. The method relies on selecting RI peak which is free from interference so that it can be used to establish a calibration curve to determine the concentration of each biopolymer in mixture. Stock solutions of EM10 and HA were mixed at different proportions and were used to establish a phase diagram. The mixtures were centrifuged for 3 h at 25°C and 4000 rpm using a Heraeus Megafuge 1.0R centrifuge. The mixtures were centrifuged for 3 h (which was found to be the optimum time and any prolonged centrifugation had no effect on bulk phase separation) at 25°C and 4000rpm using a Heraeus Megafuge 1.0R centrifuge. The upper and bottom phases were carefully separated by syringe and diluted to 1-2 mg/mL containing 0.2 mol/L NaCl, and injected into GPC-MALLS system for measuring the compositions of each phases.

The cloud points in the phase diagram of EM10/HA mixtures were determined by visual observation. A series of mixed solutions were prepared by fixing EM10 concentration (0.2-10.0%) and varying HA concentration (0.02-3.0%). The cloud points were determined from the observation of bulk phase separation after the mixtures were centrifuged.

#### *Effects of temperature and solvent conditions*

The effects of temperature and solvent condition on the phase separation induced molecular fractionation of gum arabic were investigated by using 10 g 3%EM10/0.25%HA aqueous solution made from 0.03 g EM10 and 0.0025 g HA. The temperatures were controlled at 4°C, 25°C, 40°C, 60°C and 80°C to figure out their

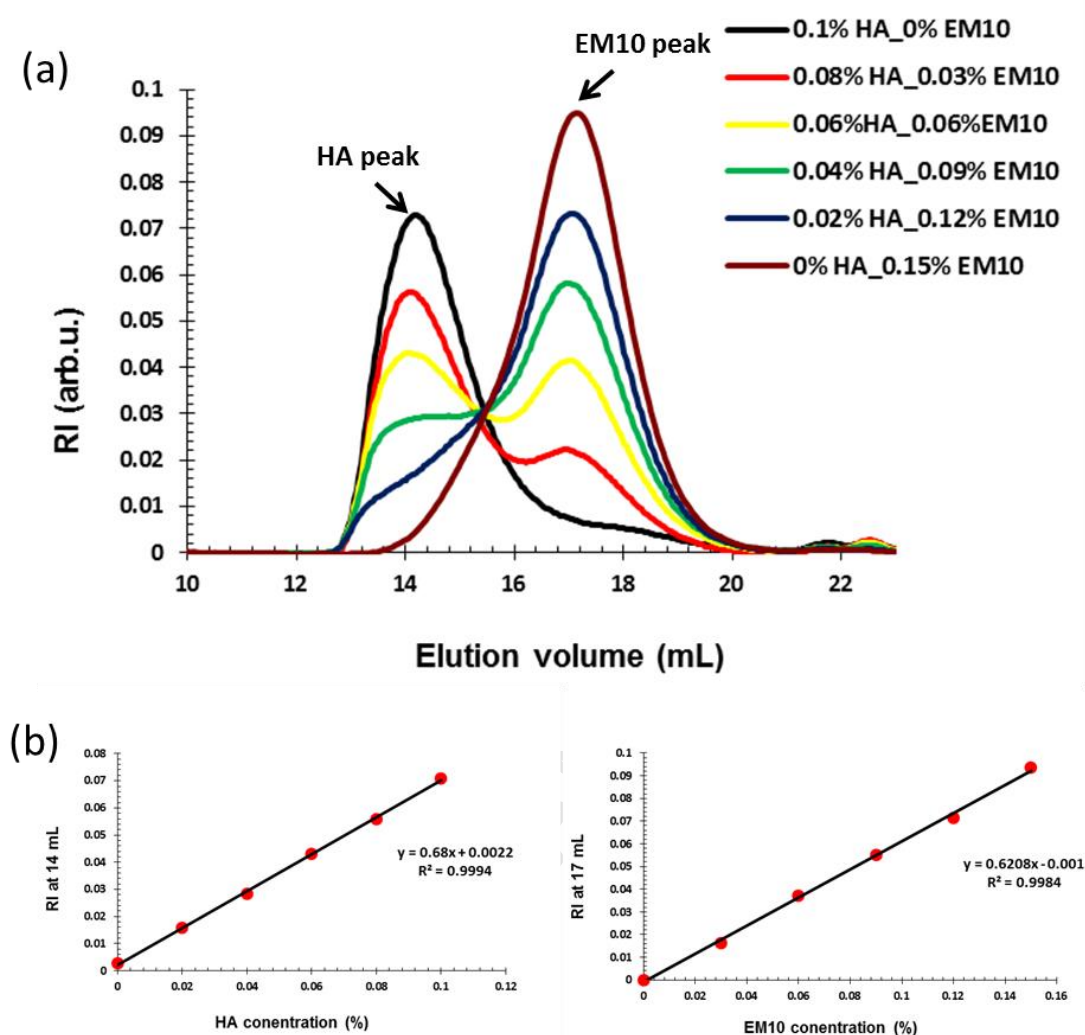
effects on phase separation. The similar 3%EM10/0.25%HA salt solutions were prepared by adding 0.03 g EM10 and 0.0025 g HA to NaCl solutions with various concentrations (0.5 – 5 mol/L) which were used to find the phase separation of EM10 and HA in the salt solution. Furthermore, the 3%EM10/0.25%HA ethanol solutions were made by adding 0.03 g EM10 and 0.0025 g HA to ethanol solutions with different concentrations (5-30% v/v) to figure out the effect of ethanol on phase separation. After centrifugation as described above, the upper and bottom phases were characterized by GPC-MALLS.

## Results and discussion

### *Phase diagram*

The experimental section introduces the procedure of establishing the phase diagram of EM10/HA mixtures by GPC-RI and visual observation. The mixing of two biopolymer solutions with different molecular weights, EM10 ( $M_w \sim 4 \times 10^6$  g/mol) and HA ( $M_w \sim 1.6 \times 10^6$  g/mol), results in a compatible or separate phase system depending on the concentration regimes utilized. A phase separated system is usually characterized by appearance of turbidity in the solution either immediately or after standing. Such a system will separate into two layers following centrifugation.

Fig. 1a shows that EM10 and HA were eluted separately at different elution volumes under the current experimental condition. The RI peak intensity was used to establish the calibration curves for EM10 (at 17 mL) and HA (at 14 mL) by injecting various concentrations of the polymers (Fig. 1b). The calibration curves exhibit excellent linearity for both EM10 and HA, and hence were used to determine the phase composition of EM10/HA after centrifugation.

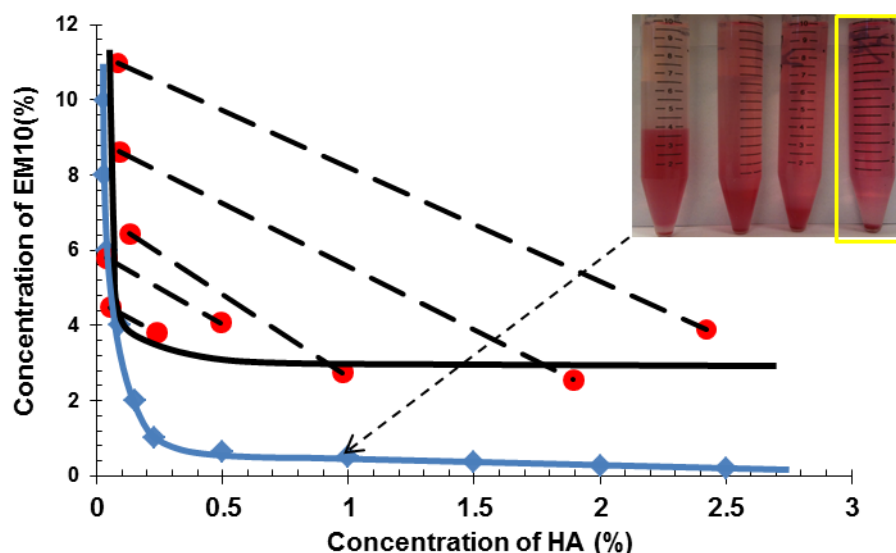


**Fig. 1. (a)** Elution profiles of EM10/HA mixed solutions containing various proportions of the two polymers, as obtained by GPC-RI. **(b)** Calibration curves of HA and EM10 established using GPC-RI.

Fig. 2 shows the binodal curve in phase diagram of EM10/HA determined by the GPC-RI method using a series of EM10/HA mixtures of various initial concentrations. A pair of red circles, joined by a dashed line (tie line), represent the composition of the two layers of a phase separated mixture. Therefore, the binodal curve (red circles fitted by a solid line) is the phase boundary which marks the compatible region and the phase separated region. According to the phase diagram, EM10 and HA are only compatible when the EM10 concentration is below around 2.0%, or when the HA concentration is extremely low, that is, less than around 0.05%. The threshold concentration of phase



separation is much lower for HA than that for EM10, although the latter has a larger average molecular weight. This should be ascribed to the linear conformation of HA in comparison to the branched and sphere-like conformation of EM10 [19, 39-41].



**Fig. 2.** A comparison of the phase diagrams of EM10/HA mixtures at 25°C obtained by GPC-RI (●) and visual observation (◆) methods. The dashed lines are tie lines which characterize the compositions of pairs in the separated phases. An example of measuring cloud points by visual observation was provided as inset.

In addition to the binodal boundary, the phase diagram of EM10/HA was also investigated by determining cloud points *via* visual observation. The cloud points were determined from the observation of bulk phase separation after the mixtures were centrifuged at 4000 rpm for 3 h and were judged by the disappearance of the separated phase, which is exemplified in the inset of Fig. 2. Fig. 2 compares the cloud points (blue diamonds) to the binodal curve at 25°C. It is clear that the cloud points do not reach and overlap with the binodal curve. The difference is too large to be due to measurement errors. Similar behavior was observed in other systems such as poly (ethylene oxide)/dextran [17], gelatin/dextran [11], maltodextrin/agarose [42], and gum arabic/sugar beet pectin [19] systems. The phenomenon has been interpreted to be due

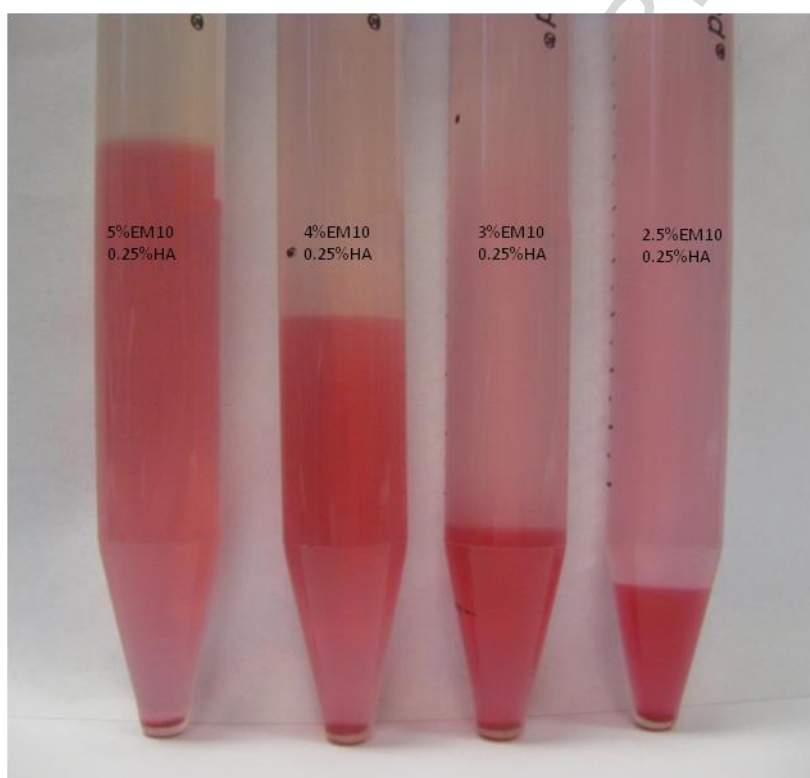
to a phase separation induced molecular fractionation of polydisperse biopolymers. Moreover, the deviation of binodal curve from the cloud points is greater in terms of EM10 concentration than HA concentration. This could be explained by a larger extent of fractionation of EM10 than HA, due to its higher polydispersity and heterogeneity, as discussed below.

It has been widely acknowledged that phase separation is strongly influenced by the molecular weight of biopolymers which results in a higher tendency to phase separation with increase of the molecular weight [12]. EM10 and HA samples used in this study have different molecular weights with polydispersity index values of 8 and 2.5, respectively. Consequently, the fraction of higher molecular weight tends to segregate while the lower molecular weight fraction has relatively higher tolerance to coexistence [12]. This is the basic perspective of phase separation induced molecular fractionation. Furthermore, GA is heterogeneous polymer consisting of three molecular species, namely, AGP, AG and GP [26, 43]. These components have distinct chemical structures and molecular weights. HA is an extracellular matrix component and a high molecular weight glycosaminoglycan composed of disaccharide repeating units of N-acetylglucosamine and glucuronic acid [44]. The phase separation of EM10/HA should, therefore, be treated as a multi-component system rather than a classic binary system. The deviation between binodal points and cloud points could, therefore, be attributed in part to the complex components contained in EM10 and HA, in addition to their broad molecular weight distributions [12].

#### *Phase separation induced fractionation*

The phase separation induced molecular fractionation was quantified upon varying the extent of phase separation. Fig. 3 shows the images of EM10/HA mixed solutions at various ratios. The solutions contained a fixed concentration of HA at 0.25% and a decreasing concentration of EM10 from left to right: 5%; 4%; 3% and 2.5%. The solutions were stained with Direct Red dye to increase the contrast between layers. Direct Red Dye 23 is an azo dye that has been used as a model compound in adsorption

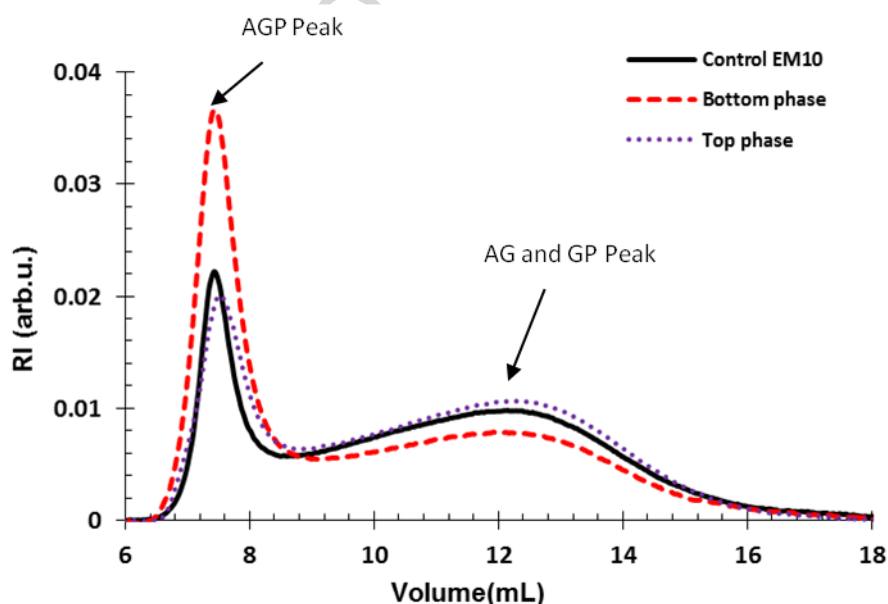
studies and utilized to compare efficacy of textile dye removal in aqueous solutions [45]. The binding of azo dyes to gum arabic has been recently reported [46]. Visual inspection of the phase volume of separated systems did not show any difference in the presence and absence of Direct Red dye 23 and thus the dye merely served to increase the contrast and identify gum arabic rich phase. Clearly the proportion of the EM10 rich layer (the bottom layer) decreases monotonically with decreasing EM10 concentration. It can be seen that relative to the bottom phases, the upper phases are depleted in gum arabic but rich in hyaluronan.



**Fig. 3.** An image showing the control of phase separation extent between EM10 and HA by varying mixing ratios (5%EM10/0.25%HA, 4%EM10/0.25%HA, 3%EM10/0.25%HA and 2.5%EM10/0.25%HA (from left to right) stained with 0.05% Direct Red dye 23).

As can be seen in Figs 2 and 3, the extent of phase separation can be adjusted by varying the mixing ratio of EM10 and HA. Here, the effect of the phase separation extent on molecular fractionation was investigated using GPC-MALLS system. The upper and bottom phases were separated carefully by using a syringe needle to

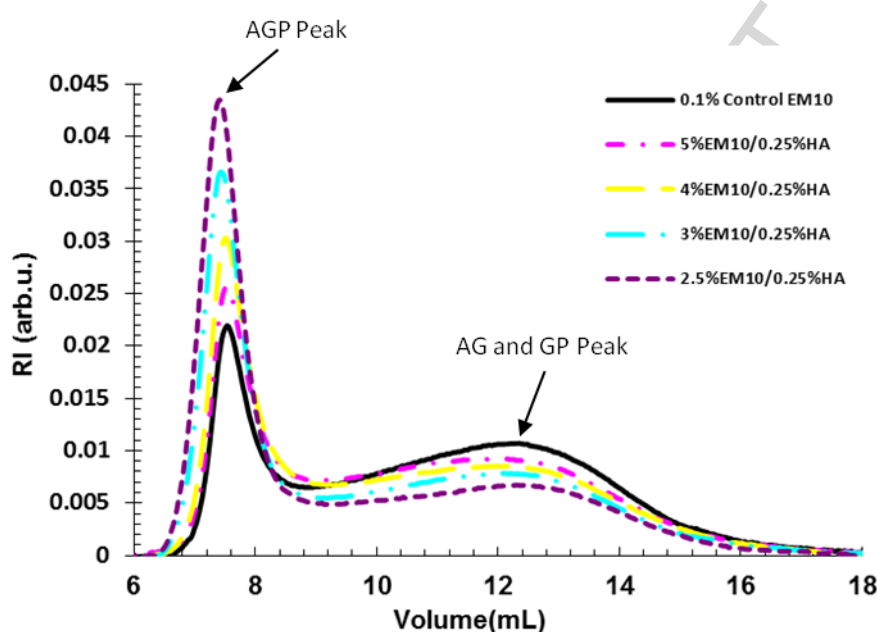
withdraw a sample from the respective layers. The sample was then diluted and injected into GPC-MALLS system to determine molecular parameters. Fig. 4 shows typical RI elution profiles for two products obtained respectively from the upper and bottom layers of a phase separated mixture containing 0.25% hyaluronan and 3% EM10. The EM10 control sample prepared directly in the mobile phase was also measured for comparison. All the RI curves obtained had two peaks: the first one is known to be associated with the AGP fraction while the second corresponds to the AG and GP fractions [25]. In comparison with the EM10 control sample, the product obtained from the bottom phase (EM10-rich phase) has an increased signal of AGP peak but a decreased signal of AG and GP peaks. On the other hand, the product obtained from the upper phase (HA-rich phase) has a decreased signal of AGP peak but an increased signal of AG and GP peaks. Similar results were observed in all the phase separated systems of EM10 and HA. Therefore, it can be concluded that the new series of products obtained from the bottom layers are AGP-rich EM10 products, and those from the top layers are AG and GP rich products.



**Fig. 4.** RI profiles in GPC-MALLS measurements for the control EM10, the bottom layer and top layer of the phase separated mixture of 0.25% hyaluronan and 3% EM10.

Fig. 5 shows the RI profiles in GPC-MALLS measurements for the starting EM10

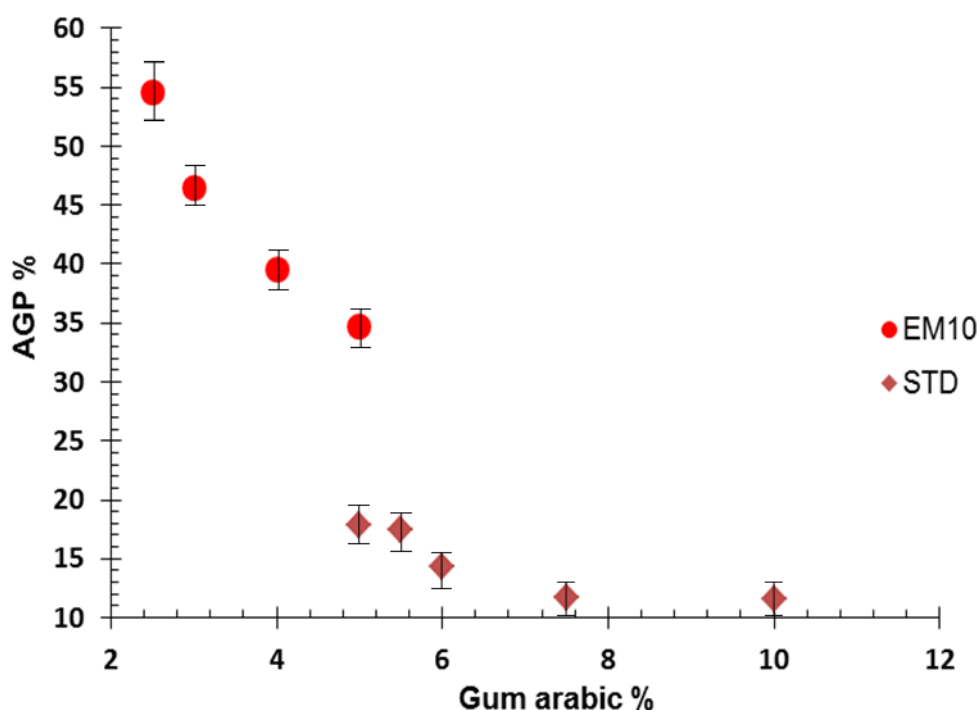
and a series of AGP-rich gum arabic products obtained *via* different extents of phase separation. Clearly, the AGP peak at ca. 7 mL increases with decreasing EM10 concentration in the mixtures, while the AG and GP peak at ca. 12 mL decreases simultaneously. The results clearly demonstrate that the AGP content can be controlled by adjusting the phase separation extent.



**Fig. 5.** RI profiles in GPC-MALLS measurements for the control EM10 and a series of AGP-rich EM10 products obtained *via* different extents of phase separation with HA.

Fig. 6 shows the variation of AGP content as a function of gum arabic (EM10 and STD) concentration upon phase separation induced molecular fractionation at a fixed HA concentration of 0.25%. The AGP content increased from 34 % to 55% when the EM10 concentration was decreased from 5% to 2.5%. The AGP content of standard gum arabic also increased from 11% to 18% when the concentration of STD was decreased from 10% to 5%. The extent of increase in AGP content is much more significant for EM10/HA system than STD/HA system. Moreover, a much higher concentration was required for STD than EM10, in order to initiate phase separation and the accompanying molecular fractionation. Segregation becomes stronger with increasing molecular weight of either of the polymeric components as noted in the

previous studies [47-49], which explains the better fractionation when using the higher molecular weight EM10.

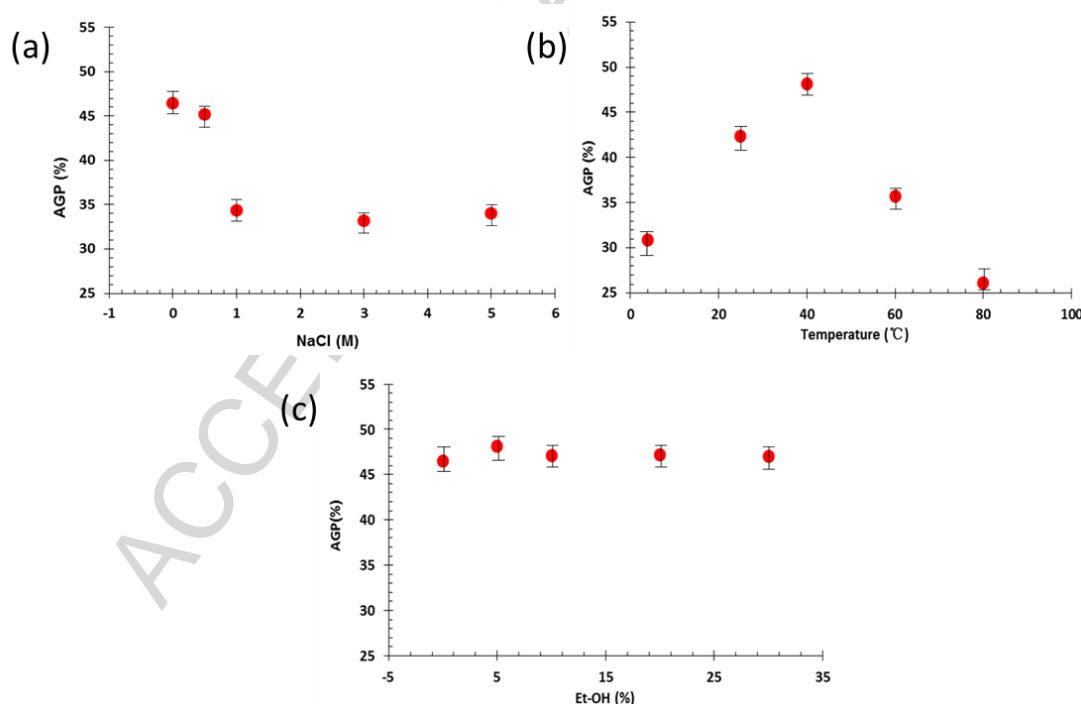


**Fig. 6.** AGP content (with error bar) of a series of AGP-rich gum arabic products obtained *via* different extents of EM10 and STD phase separation with HA.

#### *Effect of salt, ethanol and temperature*

The ionic environment of a mixture often influences the phase behavior of a mixed system. Fig. 7a shows the effect of NaCl concentration on the phase separation induced molecular fractionation for the mixture of 3%EM10/0.25%HA. The AGP content of EM10 in the bottom phase is plotted against NaCl concentration. There was a small decline in AGP content when NaCl was increased to 0.5 M, but it sharply decreased and leveled off at NaCl > 1 M. The formation of liquid/liquid phase separation between GA and HA was found to be mainly dominated by electrostatic repulsive forces. The tendency of phase separation was related to the strength of the electrostatic interaction between GA and HA molecules [50], and the NaCl present in the solution could decrease the yield of phase separation induced fractionation due to reduced repulsive

force between GA and HA. Hence, the salt increases the miscibility and restrains the phase separation of EM10/HA mixed system and subsequently the molar mass fractionation. The effect is ascribed to changes in the degree of ionization and shielding of the acid groups, and hence changes in the average distance between charged points in the polymer chain. Similar results about the effect of salt addition in whey protein isolate-pectin phase separation system were also documented by Thongkaew et al. [51]. Therefore, in a mixture of polyelectrolytes, a raised ionic strength is able to shield electrostatic effect, and in the case of two similarly charged polyelectrolytes a reduction in the repulsion is possible, thereby reducing the likelihood of separation or raising the critical concentration threshold. The situation is more complex when only one of the polymers is charged. If a salt is added to such a system, the increase in the ionic strength will promote phase separation to occur because the counterion entropy effect is masked by that of the added salt [52-54].



**Fig. 7.** Variation of AGP content (with error bar) of the AGP-rich EM10 products obtained from mixing 3%EM10/0.25%HA at different concentrations of NaCl (a), different temperatures (b) and different ethanol contents (c).

Temperature is also a critical factor that affects the phase separation. Fig. 7b shows the effect of temperature on the phase separation induced molecular fractionation for the mixture of 3%EM10/0.25%HA. Increasing the temperature favored the occurrence of phase separation but such a behavior vanished at 80°C. AGP content changed from low to high temperatures in a way similar to a parabolic curve. The optimal fractionation temperature was found to be around 40°C. It is necessary to point out that the AGP content at 80°C is only 26%, which is even lower than that of the control EM10. It is likely that the high temperature may induce the structural breakdown of the polymer [55]. Table 1 shows clearly that the molecular weights of EM10 and HA in mixed solutions have decreased after 24 h at 80°C in the water bath. This also confirms the above phase separation behavior vanished at 80°C. The molecular weights for the various mixtures reduced almost by half after 24 h at 80°C. Hence, a decrease of the molar mass for both EM10 and HA with increasing temperature is observed, which is probably due to temperature-induced hydrolysis of EM10 and HA [56]. In addition, increasing temperature to 40°C leads to an increase in interaction between solvent molecules and EM10 and HA, respectively, and hence a decrease in compatibility between EM10 and HA. This would intensify segregative phase separation between EM10 and HA [57], and explain the increased AGP content up to 80 °C. Further increasing temperature to 80°C would increase the compatibility and reduce segregative phase separation between EM10 and HA, due to reduction in molecular weights caused by temperature-induced hydrolysis [58]. Therefore, APG content was decreased with increasing temperature in the higher temperature range. Hence, to make use of the phase separation of EM10/HA mixture solution the best temperature is around 40°C, which assures a good yield volume and highest AGP content. However, the optimal temperature in phase behavior is not a universal law in hydrocolloid mixtures. Edelman *et al.* [11] studied the phase separation-induced fractionation in aqueous mixtures of gelatin and dextran and showed that temperature had no effect on final molar distributions after phase separation.



**Table 1** Molecular weight of EM10 and HA in mixed solutions before and after 80°C.

Mixture solutions	Mw of EM10 ( $10^6$ Da)		Mw of HA ( $10^6$ Da)	
	Before 80°C	After 80°C	Before 80°C	After 80°C
5%EM10/0.25%HA	4.011	2.256	1.685	0.812
4%EM10/0.25%HA	4.095	2.134	1.678	0.806
3%EM10/0.25%HA	4.074	2.226	1.677	0.786
2.5%EM10/0.25%HA	4.063	2.103	1.688	0.754

Fig. 7c shows the AGP content of AGP-rich EM10 products from mixing 3%EM10/0.25%HA obtained *via* different concentrations of ethanol. It is clearly seen that the ethanol has no influence on the phase separation and molar mass fractionation of mixed EM10/HA system and the AGP content does not change with increasing concentration of Et-OH. Ethanol at critically high concentrations is a common anti-solvent (precipitant) for hydrocolloids [59]. For gum arabic, it is soluble in dilute alcohol solution but starts to precipitate out when the alcohol concentration in water is about 50%, with a complete precipitation of gum arabic macromolecules with 60% alcohol [60]. As is well known for gum arabic, it is the protein-rich high-molecular weight fraction, the AGP complex, that mainly provides the emulsification properties due to the hydrophilic carbohydrate blocks and hydrophobic polypeptide chain in the structure [61]. Consequently, the concentration of ethanol in solvent has only a slight influence on phase separation due to high AGP content in EM10 that have more hydrophilic group to adapt to ethanol solvent. It explains that the EM10 has a good stability in relatively high concentration ethanol. It also explains that the HA has a good stability in ethanol solvent although HA is used only at very low concentrations. The

similar study on higher ethanol concentrations in EM10/HA mixture will be done in the future work.

Thermodynamic incompatibility underlying phase separation largely comes from the entropy of mixing (excluded volume effect) [62], which again depends on the molecular weight of the biopolymers in the mixed solution. Some previous experiments showed that the high temperature (80°C) induced degradation of GA and HA due to temperature-induced hydrolysis. Chikamai *et al.* investigated the effects of heating on gum arabic solutions at 100°C and 65°C. They reported that heating the gum solution at 100°C for more than 6 h caused a significant degradation of the protein component while less effect was observed at 65°C [58]. Therefore, a lower molecular weight results in a weaker tendency toward phase separation, and the optimal fractionation temperature was found to exist around 40°C. Dror *et al.* investigated the structure of GA solutions at different salt concentrations by small-angle X-ray and neutron scattering combined with cryo-transmission electrons microscopy [63]. A scattering peak was observed at moderate to high concentrations, the spacing of which exhibited a  $c^{-1/3}$  power law relation to polymer concentration ( $c$ ). Upon addition of salt (0.5M NaCl), this peak disappeared, indicating its electrostatic screening effect. Added salt had slightly effect on the molecular weight of GA below 0.5M NaCl and did not affect significantly the molecular weight of GA as the salt concentration increased (0.5-5M). Hence, segregative phase separation receded mainly due to the electrostatic interaction being screened by salt. Ethanol precipitation is one of the most widely used methods for preparing natural polysaccharides, which however, is usually set at 70–80% [64]. Bouchard *et al.* investigated the solubility and viscosity of sugars, polyols and polysaccharides in water and water-ethanol mixtures [65]. They reported that the increase in ethanol fraction caused a decrease in solubility in all cases. However, the present study showed that an ethanol concentration below 30% v/v has negligible effect on segregative phase separation and molar mass fractionation of mixed EM10/HA system. This might be due to the fact that the interaction and compatibility between

EM10 and HA were nearly unaffected as a result of similar decrease in solubility between the two polymers.

## **Conclusion**

This study investigates the effects of temperature and solvent condition on phase separation induced fractionation of polydisperse GA/HA mixtures. Both STD gum and EM10 undergo segregative phase separation resulting in a significant molecular fractionation to increase the AGP content. The fractionation of EM10 depends on not only the starting mixture concentration, but also the temperature and solvent conditions. Temperature and addition of salt show a significant influence on phase separation induced fractionation, while ethanol has almost no effect up to a concentration of 30%.

## **Acknowledgements**

This work was supported by Hubei Provincial Youth Natural Science Foundation (No. 2018CFB414), the state key research and development plan "modern food processing and food storage and transportation technology and equipment" (No. 2017YFD0400200), National Natural Science Foundation of China (31671811), and Wuhan Science and Technology Bureau (2015070504020218).

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